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Isolation and analysis of chitinase from marine bacteria

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Abstract
Chitinase is involved in the process of producing mono and oligosaccharides from chitin. Furthermore, chitinase is a potential antifungal agent through its chitin degradation activity. Chitinase producing marine bacteria play an important role in the degradation of chitin in the ocean. In contrast chitin is absent in vertebrate animals and higher plants. Chitinivorous organism include many marine bacteria (Aeromonads, Bacillus, Vibrio among others). Chitinase have been implicated in plant resistance against fungal pathogens because of their inducible nature and antifungal activity *in vitro*. Isolation of marine Bacillus species from seawater by serial dilution and the bacteria has enzymatic activity. Although, chitinase have been isolated and characterised from a wide variety of sources, it is still important to screen for new sources for the production of chitinase with enhanced property and more economical values for the purpose of expanding their usefulness.

Keywords: chitinase, bacillus, isolation

1. Introduction
Chitin is a polymer of β-1, 4 linked N-acetyl glucosamine (GlcNAc) and a very abundant natural polymer. Chitin is a structural homologue to cellulose It is the main compound of cell wall of fungi, insect exoskeleton and the shell of crustaceans. Chitinhydrolysate can be used as the carbon and nitrogen source in the production of single cell protein. Chitinivorous organisms include many bacteria (*Aeromonads, Bacillus, vibrio* among others), Which may be pathogenic or detritivorous. The attack living arthropods, zooplankton or fungi or they may degrade the remains of these organisms. Fungi, such as *Coccidioides immitis*, also posses degradative chitinases related to their role as detritivores and also to their potential as arthropod pathogens. Chitinases are also present in plants; some of these are pathogen related (PR) proteins that are induced as part of systemic acquired resistance. Chitinase were also reported in gastric juices of human being (Paoletti *et al*., 2007) [3] where they were being thought to be involved catabolic activities. Further chitinase activity was also detected in human serum (Escott and Adams David, 1995) [2] and found very similar to plant chitinases those are related in the process of information and pathogen resistance (Chupp *et al.*, 2007) [1]. Chitinase producing marine bacteria play an important role in the degradation of chitin in the ocean.

2. Materials and Methods
2.1 Sample Collection
The microorganism that produce chitinase enzyme was isolated from marine water.

- **Gram stain-reagents**

Crystal Violet
Distilled Water
Gram's iodine
Alcohol
Saffranin

- **Nutrient broth- composition (pH 7)**

Peptone
NaCl
Beef Extract
Distilled water

- **Nutrient agar- composition (pH 7)**

Peptone
NaCl
Yeast extract
Agar
Distilled water

- **CCA plate**

Colloidal chitin
Nutrient Agar

- **Colloidal chitin broth**

Colloidal chitin
Nutrient Broth

2.2 Chemicals used for biochemical identification
- Methyl red indicator for Methyl red test.
- Barritt's reagent A & B for Voges Proskauer Test
  Reagent A is prepared by sissolving 6 grms of a-naphthalene in 100 ml of 95% ethyl alcohol.
  Reagent B is prepared by dissolving 16 grams of potassium hydroxide in 100 ml of water.
- Kovacs reagent used for Indole production test
  Kovac's reagent is a biochemical reagent consisting of isoamylalcohol, para-dimethyl amino benz aldehyde and concentrated hydrochloric acid. It is used for the

diagnostical indole test, to determine the ability of the organism to split indole from the aminoacid tryptophan.
- 1% Tetra methyl paraphenylene diamine hydrochloride is used as test reagent in oxidase test.
- H_2O_2 for Catalyse test.

2.3 Chemicals used for protein estimation
- Reagent A-2% Na_2CO_3 in 0.1 N of NaOH.
- Reagent B- 0.5% $CuSo_4$ in 1% Potassium sodium tartarate.
- Reagent C-Alkaline copper solution.
- Reagent D – Folin ciocalteau reagent.

2.4 Isolation of Marine Bacteria from Sea Water
Sea water were collected from coastal area of Cochin. 1 ml of the sample was mixed with 9 ml of sterile distilled water and shaken well. 1 ml of solution was pipetted out and added to another test tube containing 9ml distilled water. 1ml of the solution was taken from the second test tube and added to another test tube containing 9 ml distilled water. This process was continued till a dilution of 10 was obtained. From the final dilution, 0.1 ml was pipetted out into plates containing selective media (Colloidal chitin plate).The plate was incubated at an inverted position at 37ºC for 24-48 hours.

2.5 Morphological and microscopical examination of bacteria
The morphological analysis of each isolate was performed in order to check the purity of isolates. The colony characteristics were analysed by simple streaking of the bacteria on nutrient agar medium (pH 7).

- **Gram staining**

Preparation of smear
A dust free and oil free microscopic glass slide is taken. Small amount of young culture is inoculated to the slide and spread uniformly. The slide is heat fixed by passing the slide 3-4 times to the flame.

Staining
Crystal violet was added on a thin smear drop by drop and spread it throughout the smear and it was kept for 1 minute.The slide was washed with distilled water. Gram's iodine was added and it was kept for 1 minute. The slide was washed with distilled water. Saffranin was added and waited for 20-30 seconds. The slide was washed with distilled water. After air dry a drop of oil was added on the smear. The slide was observed under microscope using oil immersion objective (100x).

- **Preparation of slant**

Two test tubes were taken and about ¼ of the test tubes were filled with nutrient agar and placed in slanting position and allowed to solidify. Care must be taken while slanting the test tube; the nutrient agar should not touch the cotton plug. The zigzag streaking was done inside the tube using pure bacterial isolate. And waited 24 hours for the inoculums to grow.

- **Effect of chitinase**

Colloidal chitin preparation
The chitin flakes were ground to powder and 2 gram of chitin powder was added slowly into 30 ml of concentrated HCl under vigorous stirring. The mixture was added to 100 ml of ice cold ethanol with rapid stirring and kept overnight 25º C. The precipitate was collected by centrifugation at 1000 rpm for 5 minutes and washed with distilled water until the colloidal chitin become neutral pH 7.

Screening for chitin degradation
CCA plate was prepared by adding colloidal chitin to nutrient agar and the agar was allowed to solidify. The microbial culture was spotted on the CCA plate using sterile inoculation loop. The plate was incubated at 37 ºC. After 24 hours the plate was observed for zone of clearance.

2.6 Biochemical examination
Methyl red test
2 test tubes were taken with nutrient broth and labelled one as methyl red and other as control tube for methyl red test. And inoculums of 200 µl were added to test tube labelled as methyl red and no inoculums were added to control tube. After incubation (24 hours), added a few drops of methyl red indicator into the broth. The colour change was observed.

Voges proskauer test
2 test tubes were taken with nutrient broth and labelled one as Voges test and other as control tube for Voges test. And inoculums of 200 µl were added to test tube labelled as Voges test and no inoculums were added to control tube. After incubation period (24 hours), few drops of Barritt's reagent A & B we were added to each test tube. The colour change was observed.

Indole production test
2 test tubes were taken with nutrient broth and labelled one as Indole test and other as control tube for Indole test. And inoculums of 200 µl were added to test tube labelled as Indole test and no inoculums were added to control tube. After incubation period (24 hours), Kovacs reagent was added. The colour change between test & control test tubes was observed.

Oxidase test
After incubation (24 hours), a small bit of filter paper were dipped into 1% test reagent and a loop full of culture was smeared on it. The result was noted.

Calalyse test
After incubation (24 hours), a small bit of filter paper were dipped into 1% test reagent and a loop full of organism was added on to the H_2O_2 to the slide. Then a loop full of organisms was added on to the H_2O_2 solution with the help of a sterile inoculation loop. The result was noted.

Protein Estimation
Lowry's method is commonly used for the estimation of proteins. Pipette out 0.2, 0.4. 0.6, 0.8 and 1 0f working standard into a series of test tubes. Then pipette out 0.1&0.2 of the sample extract two other test tubes. A tube with 1 ml of water served as the blank. Then added 5 ml of reagent C to each test tube including the blank. Mixed well and allowed it to stand for 10 minutes. Then added 0.5 ml of reagent D

mixed well and incubated at room temperature in dark for 30 minutes. Blue colour developed. The OD was taken at 660 nm.

3. Results and Discussion

- **Isolation**

After incubation period single colonies with clear zone were observed on the CCA plate.

- **Screening**

Streaking

Pure white colonies of bacteria are formed on petri plates and slants after incubation for 24 hours.

Gram Staining

Microscopic examination of isolated bacterial colonies following staining revealed that the given bacteria were rod shaped and gram positive. The given bacteria being gram positive was indicative of low lipid content in the cell wall. The crystal violet stain persisted even after washing with alcohol and adding of safranin. Due to this the bacteria were stained violet.

Screening for chitin degradation

The chitin present in the CCA plate was degraded by the chitinase enzyme produced by the bacteria. Due to the activity of chitinase, a zone of clearance measuring 5 mm was produced around the bacterial growth.

- **Biochemical Assay**

Methyl red test

No bright red colour was obtained which shows a negative result for methyl red. The microorganisms doesn't have the ability to oxidase glucose and produces high acid concentration.

Voges proskauer test

Absence of pink or deep rose colour indicates negative reaction for the test. The bacterium doesn't have the capacity to produce non acidic or neutral end products.

Indole production test

Formation of cherry red ring will take place on top of test tube in indole containing incubation tube and in control tube a greenish colour is obtained. Indole production takes place by the degradation of tryptophan present in the bacteria.

Oxidase test

A violet colour obtained on filter paper indicates positive result to the test. Bacteria have the ability to produce Cytochrome C oxidase. The 1% test reagent added gets oxidised to form violet or purple colour on filter paper and it is known as Water's Blue.

Catalyse test

Bubble formation takes place in the inoculated tube and no change in the control tube. Bubble formation gives a positive result which indicates that H_2O_2 is degraded into water and free oxygen gas is liberated.

Protein estimation

Table 1: Protein content was calculated by taking OD at 660 nm.

Test tube	OD at 660 nm
BLANK	0
0.1	0.08
0.2	0.29

Table 2: Standard value of BSA at different OD

OD at 600 nm	Concentration of BSA (mg/ml)
0.34	40
0.68	80
1.02	120
1.20	160
1.32	200

Chitinase is a group of enzymes which are capable of degrading chitin polymer into N-acetyl giucosamine by either endo or exo cleavages of the 1-3 and 1-4 bond are found in organisms including bacteria (also marine bacteria), higher plants, insects, crustaceans, invertebrates and some vertebrates. This study shows the isolation, screening and characterization of marine bacteria producing chitinase. After isolated the gram-positive rod shaped chitinase producing bacillus species biochemical tests the bacteria was found to be methyl red test negetive, voges proskauer test negative, indole production test positive, oxidase test positive, catalyse test positive. By doing above studies we have done the steps towards identifying this chitinase producing bacteria. Futher biochemical activities can be tested to identify and characterize these bacteria. Marine bacillus species producing chitinase enzyme was successfully isolated by serial dilution and the bacteria obtained were screened for chitinase activity. The diameter of zone of clearance depends on the activity of chitinase enzyme. The diameter, 5 mm obtained during the experiment shows that the bacteria has enzyme activity. Chitinase isolated and characterised from a wide variety of sources and they have more economical values for the purpose of expanding their usefulness.

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5. References

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